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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

13203

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)

09/424629

TRANSMITTAL LETTER TO THE UNITED STATES
 DESIGNATED/ELECTED OFFICE (DO/EO/US)
 CONCERNING A FILING UNDER 35 U.S.C. 371

INTERNATIONAL APPLICATION NO.
PCT/AU98/00396INTERNATIONAL FILING DATE
28 May 1998 (28.05.98)

PRIORITY DATE CLAIMED

28 May 1997 (28.05.97) 30 May 1997 (30.05.97)

TITLE OF INVENTION

5 February 1998 (05.02.98) 19 May 1998

NUCLEIC ACID DIAGNOSTICS BASED ON MASS SPECTROMETRY OR MASS SEPARATION AND BASE (19.05.98)
SPECIFIC CLEAVAGE

APPLICANT(S) FOR DO/EO/US

Simon Foote Gavin Reid
Richard Simpson Colleen Elso

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371
3. This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a is transmitted herewith (required only if not transmitted by the International Bureau).
 - b has been transmitted by the International Bureau.
 - c is not required, as the application was filed in the United States Receiving Office (RO/US).
6. A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. A copy of the International Search Report (PCT/ISA/210).
8. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a are transmitted herewith (required only if not transmitted by the International Bureau).
 - b have been transmitted by the International Bureau.
 - c have not been made; however, the time limit for making such amendments has NOT expired.
 - d have not been made and will not be made.
9. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))
10. An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. A copy of the International Preliminary Examination Report (PCT/IPEA/409)
12. A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5))

Items 13 to 18 below concern document(s) or information included:

13. An Information Disclosure Statement under 37 CFR 1.97 and 1.98
14. An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
15. A **FIRST** preliminary amendment
A **SECOND** or **SUBSEQUENT** preliminary amendment
16. A substitute specification
17. A change of power of attorney and/or address letter
18. Certificate of Mailing by Express Mail
19. Other items or information

Courtesy copy of international application
Thirteen (13) sheets of drawings

U.S. APPLICATION NO. (IF UNKNOWN SEE 37 CFR 1.492)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/424629	PCT/AU98/00396	13203

20. The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
<input type="checkbox"/> Search Report has been prepared by the EPO or JPO	\$840.00	
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482)	\$670.00	
<input type="checkbox"/> No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	\$760.00	
<input checked="" type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO	\$970.00	
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	\$96.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$970.00

Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).	<input type="checkbox"/> 20	<input checked="" type="checkbox"/> 30	\$130.00
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CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	* 32 - 20 =	12	x \$18.00	\$216.00
Independent claims	* 5 - 3 =	2	x \$78.00	\$156.00
Multiple Dependent Claims (check if applicable).			<input type="checkbox"/>	\$0.00

TOTAL OF ABOVE CALCULATIONS = \$1,472.00

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).	<input type="checkbox"/>	\$0.00
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SUBTOTAL = \$1,472.00

Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).	<input type="checkbox"/> 20	<input type="checkbox"/> 30	+ \$0.00
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TOTAL NATIONAL FEE = \$1,472.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).	<input type="checkbox"/>	\$0.00
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TOTAL FEES ENCLOSED = \$1,472.00

*Claim calculation based on claims as amended by Preliminary Amendment being filed concurrently herewith	Amount to be: refunded	\$
	charged	\$

A check in the amount of **\$1,472.00** to cover the above fees is enclosed

Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No **19-1013/SSMP** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Leopold Presser
SCULLY, SCOTT, MURPHY & PRESSER
 400 Garden City Plaza
 Garden City, NY 11530
 (516) 742-4343

SIGNATURE



Leopold Presser

NAME

19,827

REGISTRATION NUMBER

November 24, 1999

DATE



SITION FEE

Colleen ELSO

Applicant or Patentee: Richard SIMPSON & Gavin REID Attorney: _____
 Serial or Patent No.: _____ Docket No: _____
 Filed or Issued: _____
 For: "Nucleic acid diagnostics based on mass spectrometry or mass separation and base specific cleavage"

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH
 ADDRESS OF ORGANIZATION Royal Parade, Parkville, Victoria, 3052,
Australia

TYPE OF ORGANIZATION

UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
 TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c)(3))
 NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
 (NAME OF STATE _____)
 (CITATION OF STATUTE _____)
 WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) AND 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
 WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
 (NAME OF STATE _____)
 (CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled Same as above by inventor(s) described in
Same as above

the specification filed herewith
 application serial no. _____, filed _____
 patent no. _____, issued _____

National Phase Entry of International Patent Application No. PCT/AU98/00396 filed 28 May 1998

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention overriding to their status as small entities. (37 CFR 1.27)

NAME _____

ADDRESS _____

INDIVIDUAL SMALL BUSINESS CONCERN NONPROFIT ORGANIZATION

NAME _____

ADDRESS _____

INDIVIDUAL SMALL BUSINESS CONCERN NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Dr Margaret Brumby - General Manager

TITLE IN ORGANIZATION The Walter and Eliza Hall Institute of Medical Research

ADDRESS OF PERSON SIGNING Royal Parade, Parkville, Victoria, 3052,

Signature: Margaret Brumby

Date: 17.02.2001

09/424629

514 Rec'd PCT/PTO 24 NOV 1999

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Simon Foote et al.

Examiner:

Serial No.: to be assigned

Art Unit: unassigned

Filed: concurrently herewith

Docket: 13203

For: NUCLEIC ACID DIAGNOSTICS

Dated: November 24, 1999

BASED ON MASS SPECTROMETRY OR MASS
SEPARATION AND BASE SPECIFIC CLEAVAGE

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

In connection with the filing of the above-described application, kindly enter the following preliminary amendments.

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

"Express Mail" Mailing Label Number: EM341609046US
Date of Deposit: November 24, 1999

I hereby certify that this Preliminary Amendment is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. §1.10 in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: November 24, 1999

Mishelle Spina

AMENDMENTS

IN THE CLAIMS:

Please amend claims 3, 6, 8, 12, 15, 17, 26, 29 and 31 as follows:

Claim 3, line 1, please delete "or 2";

Claim 6, line 1, please amend "any one of claims 1 to 5" to read --claim 1--;

Claim 8, line 1, please amend "any one of claims 1 to 7" to read --claim 1--;

Claim 12, line 1, please delete "or 10";

Claim 15, line 1, please amend "any one of claims 9 to 13" to read --claim 9--;

Claim 17, line 1, please amend "any one of claims 10 to 16" to read --claim 10--;

Claim 26, line 1, please delete "or 25";

Claim 29, line 1, please amend "any one of claims 24 to 28" to read --claim 24--; and

Claim 31, line 1, please amend "any one of claims 24 to 30" to read --claim 24--.

REMARKS

As originally prepared, Claims 6-9, 15-18 and 29-32 did not comply with the multiple dependent claim style

specified by U.S. law. The amendments submitted herewith have been made to eliminate all multiple dependencies. It is respectfully submitted that no new matter has been introduced by so doing.

It is respectfully requested that the above amendments be entered before an action on the merits is issued.

Respectfully submitted,



Leopold Presser
Registration No. 19,827

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LP/vjs



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09/424629

514 Rec'd PCT/PTO 24 NOV 1999

WO 98/54571

PCT/AU98/00396

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NUCLEIC ACID DIAGNOSTICS BASED ON MASS SPECTROMETRY OR MASS SEPARATION AND BASE SPECIFIC CLEAVAGE

FIELD OF THE INVENTION

5 The present invention relates generally to a method for detecting a mutation in a nucleic acid molecule. The method of the present invention does not require prior knowledge of a reference or wild-type nucleotide sequence nor does it require a gel electrophoresis step. The method of the present invention is particularly useful in identifying mutations and polymorphisms in genomic DNA and more particularly in the human genome and to determine and/or confirm the
10 nucleotide sequence of target nucleic acid molecules. The method of the present invention may also be automated.

BACKGROUND OF THE INVENTION

15 Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

The increasing sophistication of recombinant DNA technology is greatly facilitating research and development in a range of biotechnological fields. A particularly important area is the generation
20 of nucleotide mutants and the screening for and identification of such mutants. This in turn has implications, for example, in understanding the genetic basis behind certain disease conditions which is becoming of increasing relevance as the human genome is progressively sequenced.

An efficient and accurate method of mutation detection is crucial in implicating disease candidate
25 genes and in the screening programs which follow identification of disease causing mutations. Many human inherited and sporadic disorders are caused by small mutations including base substitutions, additions and deletions. Among these disorders are the Mendelian single gene disorders, sporadic somatic mutations causing cancers and complex genetic traits. Whilst some diseases are caused by a limited and well characterised set of mutations, most genetic diseases
30 are caused by one or more of a large range of mutations occurring anywhere within the gene. It is important, therefore, that a mutation detection protocol be able to scan a region of DNA,

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identify any change and describe the resulting nucleotide differences from wild-type. With the increasing use of population molecular genetics and as clinicians begin to use mutation analysis as a clinical tool, there is a need to develop mutation detection protocols which can be automated, are less dependant on user expertise and are more accurate and reliable.

5

Current mutation detection protocols require either a gel based detection system or sequence specific primers. Gel based detection methods include direct sequencing of amplified DNA fragments and various techniques involving either cleavage of mismatched bases in heteroduplexes or mobility differences of single or partially denatured DNA strands.

10

Detection of mutations by DNA sequencing can provide good results in relation to accuracy and information about the position and nature of the mutation (Hattori *et al*, 1993), however, although advances have been made in this area, the technique is not fully automated and is labour intensive. Most mutations occur as heterozygotes and there are technical difficulties with the 15 ability of currently available computer software to identify two different nucleotide bases at a mutated residue.

Many mutation detection techniques exploit differential electrophoretic mobilities of DNA fragments with sequence differences. Single strand conformation polymorphism (SSCP) exploits 20 the fact that the secondary structure of a single strand of DNA is sequence based and, therefore, strands with even just one base difference will migrate at a different rate (Orita *et al*, 1989). This technique is again gel based and can lack sensitivity. Furthermore, the method cannot be readily automated and requires a large amount of labour due to the necessary gel step which in most cases must be optimised to the specific sample being analysed. They also do not give any 25 information about the position or nature of the change and do not routinely identify all mutations.

Mutation detection based on the identification of base pair mismatches in heteroduplex DNA strands is another method of identifying point changes. There are a number of techniques available that cleave DNA at mismatched base pairs in heteroduplex DNA. Mismatch cleavage 30 protocols include chemical and enzymatic mismatch cleavage. The techniques are also gel based. The chemical cleavage method uses osmium tetroxide to cleave at the mismatched base (Cotton

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et al, 1988) followed by separation of cleaved products on denaturing gels. A major disadvantage of the chemical cleavage protocol is the use of extremely toxic chemicals.

Other methods for detection of known mutations include minisequencing allele specific
5 polymerase chain reaction (PCR), oligonucleotide probe arrays (Lipshutz *et al*, 1995) which requires knowledge of the sequence of wild-type and mutant. Although this technique is suitable for non-gel based detection methods, it is only useful for known mutations. Furthermore, the large number of oligonucleotides required to cover all known mutations in many genes makes this approach prohibitively expensive and labour intensive.

10

With the development of the matrix assisted laser desorption ionisation - time of flight mass spectrometer (MALDI-TOF MS), the ability to accurately determine the mass of biomolecules of a limited size has been achieved. Although detection of DNA fragments of up to 622 base pairs in length has been reported, large fragments cannot be accurately sized and a mass accuracy
15 of ± 3 bp is quoted (Liu *et al*, 1995). This level of accuracy is clearly insufficient for the detection and characterisation of base substitutions.

There is a need, therefore, to develop an effective and accurate means of detecting mutations in nucleic acid molecules. Preferably, the mutation detection system would be automatable.

20

In work leading up to the present invention the inventors developed a mutation detection system which exploits the accuracy of mass determination of MALDI-TOF MS and which is applicable for large DNA fragments. The method of the present invention do not require gel electrophoresis nor is prior knowledge of the nucleotide sequence necessary. The method of the
25 present invention is also capable of being automated.

SUMMARY OF THE INVENTION

Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

5

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

10

One aspect of the present invention contemplates a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide 15 fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

20

Another aspect of the present invention provides a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule by polymerase chain reaction (PCR), subjecting the test amplified nucleic acid molecule to base specific cleavage to 25 generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a 30 difference of one or more nucleotides in said tested nucleic acid molecule.

Yet another aspect of the present invention is directed to a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule by PCR, subjecting the test amplified nucleic acid molecule to base specific cleavage to generate 5 oligonucleotide fragments of from about 2 to about 1000 bases, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an 10 altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Still yet another aspect of the present invention relates to a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule and incorporating uracil residues, subjecting the test amplified nucleic acid molecule to uracil specific cleavage mediated by a uracil-N-glycosylase to generate oligonucleotide fragments of from about 2 to about 1000 bases, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the 15 oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

20 Another aspect of the present invention contemplates a computer programme capable of controlling a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or 25 other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an 30

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altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

5 Yet another aspect of the present invention is directed to an apparatus capable of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said apparatus comprising means of subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent 10 procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

15

Still another aspect of the present invention provides a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide 20 fragments based on mass by MALDI-TOF MS and/or other equivalent procedure and subjecting said separated fragments to further separation means, such as post source decay (PSD) or other similar technique, to separate fragmentation products to generate a spectrum dependent on nucleotide sequence and then identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative 25 of a difference of one or more nucleotides in said tested nucleic acid molecule.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical representation showing mass spectrogram of cleavage products of two oligonucleotides, 1 and 2, which differ at two nucleotides, one produces a fragment with a different nucleotide composition and the other introducing a new cleavage site. The two line thicknesses represent the overlaid tracings of the two different oligonucleotides. 1636.3 represents a thick line peak and 3190.9 represents a thin line peak. 1811.1 is a thin line peak and 1828.2 is a thick line peak. Kratos Kompact MALDI 4v51.2; % int. 100% = 24mV (thin); 81mV (thick).

10

Figure 2 is a graphical representation showing mass spectrogram of reacted, separated products of normal TUB which represents a homozygote. Mode: linear; Accelerating Voltage: 20,000; Grid Voltage: 92.000%; Guide Wire Voltage 0-100%; Delay 125ON; Laser:1800; Scans Averaged: 128; Pressure: 9.94e-07; Low Mass Gate: 900.0; Negative Ions: ON.

15

Figure 3 is graphical representation showing mass spectrogram of reacted, separated products of both TUB-M and TUB which represents a heterozygote. Mode: linear; Accelerating Voltage: 20,000; Grid Voltage: 92.000%; Guide Wire Voltage 0-100%; Delay 125ON; Laser:1800; Scans Averaged: 128; Pressure: 1.89e-06; Low Mass Gate 900.0; Negative Ions: ON.

20

Figure 4 is a representation of the nucleotide sequence of IL-12 untranslated region PCR product used in Example 13. Primers are shown in bold. Expected cleavage products >2bp are underlined. The polymorphism is at position 97 and is indicated by asterisk. The polymorphism is a C to T change which results in a change of the cleavage products at that position from CGA 25 to AGA in the forward strand and CAAGC to CAA in the reverse stand. The presence of C at position 97 results in a TaqI site and this allele is called "+", the other allele is respectively "-".

Figure 5A is a photographic representation of a TaqI restriction digest of IL-12 PCR products from +/- individuals (lanes 1, 4 and 5), a +/+ individual (lane 3) and a -/- individual (lane 2). The 30 124 bp fragment is cleaved by TaqI (where possible) to produce 97 and 27 bp fragments.

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Figure 5B is a graphical representation showing linear MALDI-TOF spectra of cleavage products. The spectra on the left show a mass range of 1000 to 3500 and those on the right are the same spectra but show in detail the mass range from 1000 to 1700. Spectra *i* a and b are from a *-/-* individual, spectra *ii* a and b are from a *+/+* individual and spectra *iii* a and b are from a *+/-* individual. Observed masses are indicated above peaks. Arrows show the peaks that change between the two alleles.

Figure 6 is a graphical representation of the mass spectrum analysed using post source decay (PSD) on a MALDI-TOF instrument. Spectrum A is a 6mer of sequence CATCCT [SEQ ID NO:16] and spectrum B a 6mer of sequence CACCTT [SEQ ID NO:17]. Both have parent ion mass of 1727.2Da. Observed masses are shown above the peaks. PSD fragments are shown at an intensity magnification of five.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on a base specific cleavage reaction to generate a set of small oligonucleotides bounded by the base cleaved. The nucleic acid molecule may be 5 completely or only partially cleaved or digested. These fragments are then separated based on mass by MALDI-TOF MS. This generates a fingerprint of the nucleic acid fragment comprising a series of peaks where each peak represents the mass of each small cleavage product. As a result of the sensitivity of mass determination, each oligonucleotide of given length but different nucleotide composition produces a different mass. The mass of each peak, therefore, 10 corresponds to the nucleotide composition of the fragment as well as to its length. Consequently, any nucleotide substitution results in either a shifted peak due to the mass difference in the new cleavage fragment or, if the mutation changes the targeted base, a cleavage product containing a different number of bases.

- 15 Accordingly, one aspect of the present invention contemplates a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a 20 fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.
- 25 Conveniently, screening is carried out by comparing the cleavage product masses of the reference or wild-type nucleic acid to those of the test sample. Mass changes corresponding to base changes are readily observed.

Accurate mass determination of these small fragments is possible allowing unambiguous 30 assignation of base composition of each oligonucleotide. This knowledge allows deduction of the nature of the mutation and, after specific cleavage at different bases and integration of the

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data, the position of the mutation.

The method of the present invention is applicable to any nucleic acid molecule such as but not limited to DNA, genomic DNA, cDNA, plasmid DNA, satalite DNA, mRNA and other RNA molecules as well as DNA:DNA, DNA:RNA and RNA:RNA hybrids. The present invention is particularly applicable to nucleic acid molecules amplified by, for example, polymerase chain reaction (PCR).

According to this aspect of the present invention, there is provided a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule by polymerase chain reaction (PCR), subjecting the test amplified nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

20

A particularly preferred requirement is that the source of nucleic acid is cleavable to oligonucleotide fragments of from 2 bases to 1000 bases, preferably of from 3 bases to 500 bases, more preferably of from 4 bases to 100 bases and even more preferably of from 4 bases to 50 bases. Oligonucleotide fragments of form 4 bases to 40 bases are of particular usefulness 25 in practising the present invention.

Accordingly, the present invention is directed to a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule by PCR, subjecting 30 the test amplified nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments of from about 2 to about 1000 bases, separating the resulting oligonucleotide

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fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak 5 is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

The nucleic acid may be cleaved by a range of chemical molecules including enzymes. Enzymes are particularly preferred due to their specificity. One useful enzyme is uracil-N-glycosylase which cleaves DNA at uracil residues incorporated, for example, during a PCR. However, a 10 range of enzymes may be employed.

According to this embodiment, the present invention relates to a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule and 15 incorporating uracil residues, subjecting the test amplified nucleic acid molecule to uracil specific cleavage mediated by a uracil-N-glycosylase to generate oligonucleotide fragments of from about 2 to about 1000 bases, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of 20 each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

The method of the present invention is predicated in part on the fact that any oligonucleotide 25 fragment differing in nucleotide composition between mutant and wild-type (or reference) sequences will be detected. The method has advantages over previously employed techniques and such advantages include the absence of a gel electrophoresis step thereby reducing time, expertise and need for separation equipment and the lack of dependence on toxic chemicals, such as osmium tetroxide. Whilst the present invention extends to the use of such chemicals in base 30 specific cleavage reactions, it is preferred to use an enzymatic reaction to cleavage the target nucleic acid molecule.

The method of the present invention is particularly useful in detecting previously unknown mutations. This is important as a screening mechanism for inherited diseases and cancers such as during pre-natal diagnosis, diagnosis of a suspected disease or screening for carriers of disease alleles. It also has applications in polymorphism analysis of populations and in studies of evolution, drug resistance, virulence or attenuation of disease agents such as bacteria, viruses or protozoa.

The method may be carried out simultaneously or sequentially with an analysis of a reference to wild-type nucleic acid molecule. Both the test and reference nucleic acid molecules can then be compared. Alternatively, the wild-type nucleic acid molecule may already have been analysed. Conveniently, this information may be stored electronically and upon completion of the analysis of the test nucleic acid molecule, both the test and reference sequences may then be compared manually, electronically or by a computer assisted means.

15 The method of the present invention may also be used to determine the nucleotide sequence of a nucleic acid molecule.

The nucleotide sequence may be completely determined or a partial sequence obtained for example, for selected nucleotides. The method of the present invention, therefore, permits the rapid determination of a nucleotide sequence which will be invaluable, for example, in the efficient analysis of mutations.

The method of the present invention may be semi or fully automated and the present invention extends to apparatuses for automating the mutation detection assay. The apparatus may also be electronically controlled by a computer programme to facilitate the automation and/or analysis process.

Accordingly, another aspect of the present invention contemplates a computer programme capable of controlling a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide

fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same 5 procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Yet another aspect of the present invention is directed to an apparatus capable of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a 10 reference nucleic acid molecule, said apparatus comprising means of subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak 15 relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

In a particularly preferred embodiment, the method of apparatus of the present invention also 20 employs a further fragment separation means such as but not limited to post source decay (PSD). PSD, for example, uses the dissociation of highly energised ions during their flight to the detector creating a second dimension. The ions are directed into an electric field of opposite polarity and are reflected. Smaller ions are reflected earlier and reach the detector first. As the spectrum from the decay is dependent on the nucleotide sequence of an oligonucleotide rather than the 25 nucleotide composition, this avoids missing mutations in an oligonucleotide having the same nucleotide composition as a reference oligonucleotide. Although PSD is one convenient fragment separation means, the present invention extends to other similar techniques to separate fragmentation products. Generally these techniques are based on mass although may also be based on electrophoretic mobility, base size, base charge, base paring or other suitable criteria.

of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure and subjecting
5 said separated fragments to further separation means to generate a spectrum dependent on nucleotide sequence and then identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

10 The MALDI-TOF MS analysis and further separation means may be done sequentially or simultaneously.

Preferably, the further separation means includes or comprises PSD or other similar techniques to separate fragmentation products.

15

The present invention is particularly useful in identifying and/or locating mutants in heterozygotes. Mutations are detectable on both strains or on one strand only.

Yet another aspect of the present invention provides a method for identifying and/or locating a
20 mutation in one or more bases in a target nucleic acid molecule, subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a
25 reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Preferably, the separated fragments are subjected to further separation means such as but not
30 limited to PSD.

- 15 -

The present invention is further described by the following non-limiting Examples.

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EXAMPLE 1
OLIGONUCLEOTIDES

Two test 22mers oligonucleotides with two bases different were used in this study

5

CCT CAT UTT TTU TTG TAA GAG G [SEQ ID NO:1]

CCT CGT UTT TTU TTG TUA GAG G [SEQ ID NO:2]

The different bases are shown in bold.

10

For the detection of point mutations (see Example 7), the following oligonucleotides are used:

TUB:

GGT GAC CTG AAC CAC CTC GTG CGT CCA GCC GTT CGT GGC TGT CCA GTC CGC

15 GAAC TCT GAC CTG CGC AAG [SEQ ID NO:3]

TUB-M:

GGT GAC CTG AAC CAC CTC GTG CGT CCA GCC GTT CGA GGC TGT CGA GTC

CGCGAA CTC TGA CCT GCG CAA G [SEQ ID NO:4]

TUB-F:

20 GGT GAC CTG AAC CAC CTC GT [SEQ ID NO:5]

TUB-R:

CTT GCG CAG GTC AGA GTT [SEQ ID NO:6]

TUB and TUB-M are used as template DNA and differ at three residues, bolded above, which
25 comprise two point mutations and one insertion (bracketed and bolded). TUB-F and TUB-R are
the "reverse" and "forward" primers used to amplify either TUB or TUB-M in a polymerase
chain reaction.

30

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EXAMPLE 2 CLEAVAGE REACTION

The cleavage reactions were carried out using 100 pmol of oligonucleotide, 0.5 units uracil -N-
5 glycosylase (Perkin - Elmer) 1xPCR buffer (50mM KCl, 10mM Tris-HCl pH 8.3) (Perkin-Elmer)
in a 250 μ l reaction. The reaction mixture was incubated at 50°C for 20 minutes to allow
cleavage of the N-glycosidic bond at uracil. It was then heated for 15 minutes to 105°C to allow
degradation of the phosphate bonds at the basic sites. The mixture was then purified using anion
exchange resin to remove buffer salts and other impurities.

10

EXAMPLE 3 SAMPLE PURIFICATION

Qiagen Anion Exchange Resin was equilibrated in 5mM NH₄HCO₃ (Sigma) pH 8.4 (sodium
15 free). 40 μ l of the slurry was added to the reaction mixture and the DNA was allowed to bind
at room temperature for 5 minutes with gentle shaking. The beads were spun down in a bench
centrifuge and the supernatant discarded. The beads were then washed with 3x100 μ l volumes
of 5mM NH₄HCO₃ pH 8.4 (sodium free) with incubation and centrifugation between each wash.
The supernatant was discarded each time. The DNA fragments were then eluted using two 40 μ l
20 volumes of 0.5M NH₄HCO₃ pH 8.0 (sodium free), with incubation and centrifugation as before
but with the supernatant being kept. The supernatant was then evaporated to dryness on a
Savant Speedivac and resuspended twice in 20 μ l distilled water and evaporated to dryness to
remove any residual NH₄HCO₃. The final product was resuspended in 5 μ l distilled water. The
final concentration being approximately 20pmol/ μ l.

25

EXAMPLE 4 THE POLYMERASE CHAIN REACTIONS AND DNA URACIL GLYCOSYLASE REACTION

30 20 ul reactions were set up containing 2.5mM MgCl₂, 2.5 mM dATP, dCTP, dGTP, 5 mM
dUTP, 0.5U Taq Gold (Perkin Elmer), 1.5 mM each TUB-F and TUB-R oligonucleotides and

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2.4 fg or either TUB or TUB-M or a mix of both. PCR assays were incubated at 95°C for 15 minutes then cycled at 95°C - 15 seconds, 60°C - 35 seconds, 72°C - 35 seconds for 40 cycles. PCR reactions were pooled, each pool contained either 10 or 100 PCR reactions. Uracil DNA glycosylase (Perkin Elmer) was added at a ratio of 1U per 10 PCR reactions. Completeness of 5 digestion was confirmed by agarose gel electrophoresis.

EXAMPLE 5
PURIFICATION OF DIGESTED PCR PRODUCTS

10 Each DNA glycosylase reaction was loaded onto a C8 aquapore RP300 column equilibrated with 0.1M TEAA, the column washed with 0.1M TEAA at a flow rate of 0.5 ml/min and elute with 0.1M TEAA in 60% v/v CH₃CN. Peaks were collected. Column eluates were desiccated on a Savant Speedivac, evaporative centrifuge, resuspended in water to the original volume and redessicated. Pellets were resuspended in 5 ml H₂O. Mass spectrometric samples were prepared
15 as described in Example 6.

EXAMPLE 6
MASS ANALYSIS

20 3-Hydroxypicolinic acid is prepared at a concentration of 75mg/ml in 1:1 acetonitrile and water and stored at room temperature in a closed vial in the dark. A new matrix solution is prepared weekly. Cation exchange beads (Bio-Rad, 50W-X4, mesh size 100-200μm) in ammonium form were used to reduce interference from sodium and potassium adducts (Nordhoff *et al*, 1992). Samples were prepared as follows: 0.5μl matrix, 0.5μl sample (10pmol DNA) and 0.5μl cation
25 exchange resin were mixed on the slide and allowed to dry. The beads were then blown off with nitrogen gas. Samples were then analysed immediately.

Samples were run on the Kratos Kompact MALDI 4 with 337nm laser or a Perspective Voyager MALDITOF machine. Linear negative mode was used for all spectra. Fifty shots were fired at
30 power setting 70 to find a sweet spot and then a further 50 shots were fired at the sweet spot to obtain the spectrum.

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EXAMPLE 7 SIMULATION

In order to assess the ability of this technique to detect mutations, a computer simulation was 5 designed. Two different stimulations were conducted, one that models a mutation occurring in a haploid genome and the other modelling a mutation occurring in a diploid genome on the background of a wildtype sequence.

In order to optimise the detection of mutations, four separate base specific cleavage reactions 10 have been performed using separated forward and reverse strands and two different base specific reagents, in this case, thymidine and cytosine. A random library of exonic sequences has been extracted from Genbank. This comprises 100,000 kb of coding sequence concatenated into one file. Sequence strings of incremental length are removed from this file. A fingerprint for each strand is generated. This is calculated by generating the sets of post cleavage fragments for each 15 base-specific reagent and sorting the non-redundant fragments. Mutant sequences are created by mutating every residue in the wild-type sequence to each of three possible alternatives. The fingerprint of each mutant is generated and compared to the wild-type fingerprints. If the fingerprints are different, it is recorded as a successful detection and the next mutant examined. If the first base specific cleavage reaction is unable to detect the mutation on the forward strand, 20 the reverse strand is tried and so on until the reverse strand of the second reagent fails. This represents the total failure rate under the described conditions. Conceivably one could increase the power of the technique by using all four base specific reagents on both strands.

25

EXAMPLE 8 DETECTION OF BASE MUTATIONS

Overlaid tracings from the mass spectrogram are presented in Figure 1. These show the cleavage products of two oligonucleotides 1 and 2 [SEQ ID NO:1 and SEQ ID NO:2, respectively], which differ at two nucleotides, one producing a fragment with a different nucleotide 30 composition and the other introducing a new cleavage site. The new fragments resulting from these differences are easily separated. This example, observed masses deviate from calculated

by $\pm 0.02\text{-}1\%$. This is sufficient to assign the correct base composition in this case, however, it is not sufficient to blindly assign base composition peaks from a sample of unknown sequence. A study has been done which concluded that all base compositions can be uniquely specified up to the 14mer level if one base has a known composition (ie. G=1 in the case of the study, or in 5 our case, T=0) with a measurement of mass to within $\pm 0.01\%$. This is presently achievable, dependent on the mass analyser used and the sample quality and quantity (Pomerantz *et al.*, 1993).

Base specific cleavage and mass spectrometry is, therefore, able to differentiate between two 10 identical length oligonucleotides with different nucleotide compositions and hence is able to differentiate between two sequences differing at one base (Table 1). Where a mutation changes the residue involved directly in the base specific cleavage reaction (a "U" residue in the case presented here), the difference in size of the resultant products is marked (Table 1). The accuracy of mass determination allows deduction of the base composition of each fragment and 15 therefore, where the sequence is known, will enable deduction of the nature of the mutation.

Table 2 presents stimulation date for the haploid genome case and Table 3 presents the stimulation data where a mutation occurs in a diploid organism in the presence of a wild-type copy. These data are presented as cumulative "failure to identify" mutations based on both 20 strands and two base specific cleavage reactions. Therefore, the last column, which is where the "C" reaction was unable to pick the mutation on the complementary strand represents the "total failure rate" of the technique under these conditions.

EXAMPLE 9 25 DETECTION OF POINT MUTATIONS

The method of the present invention has been employed on PCR products and is able to detect point mutations and an insertion in DNA that has been amplified using the polymerase chain reaction as discussed below. The PCR templates used, TUB and TUB-M are described in 30 Example 1 and have three differences, two of which are point mutations and the third is an insertion/deletion. All of these differences are visible in the mass spectrograms (Figures 2 and

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3). Figure 3 represents the reacted, separated products of both TUB-M and TUB. This is a reconstruction of a heterozygote. Figure 2 is reacted, separated products of TUB, representing, in this case a homozygote normal. Table 4 gives the expected masses for each fragment and the corresponding comments on whether they have been seen. All mutations were seen on either
5 both strands or on one strand only.

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TABLE 1

oligo1:cleavage products		calc. mass	obs.mass
a	CCTCAT ¹	1810.2	1811.1
b	TTTT	1318.8	1318.4
c	TTGTAAGAGG ²	3190.0	3190.9
oligo2:cleavage products			
a	CCTCGT ³	1826.2	1828.2
b	TTTT	1318.8	1318.4
c	TTGT	1343.8	1343.5
d	AGAGG ⁴	1635.0	1636.3

¹ SEQ ID NO:18

² SEQ ID NO:19

³ SEQ ID NO:20

⁴ SEQ ID NO:21

Table 2.

Sequence length	Total number wildtype sequences	Total number mutated sequences	Number of "T" forward failures	% "T" forward failures	Number of "T" reverse failures	% "T" reverse failures	Number of "C" forward failures	% "C" forward failures	Number of "C" reverse failures	% "C" reverse failures
40	2500	300000	10569	3.52	97	0.03	1	0	1	0
60	1666	299880	14723	4.91	237	0.08	14	0	0	0
80	1250	300000	18908	6.30	401	0.13	26	0.01	0	0
100	1000	300000	22825	7.61	653	0.22	43	0.01	0	0
120	833	299880	26383	8.80	931	0.31	72	0.02	0	0
140	714	299880	29751	9.92	1225	0.41	93	0.03	2	0
160	625	300000	32644	10.88	1480	0.49	145	0.05	8	0
180	555	299700	35692	11.91	1955	0.65	188	0.06	11	0
200	500	300000	38848	12.95	2356	0.79	264	0.09	21	0.01
220	454	299640	40970	13.67	2828	0.94	348	0.12	24	0.01
240	416	299520	43995	14.69	3449	1.15	500	0.17	32	0.01
260	384	299520	46387	15.49	3923	1.31	541	0.18	62	0.02
280	357	299880	48443	16.15	4386	1.46	643	0.21	88	0.03
300	333	299700	50812	16.95	4904	1.64	759	0.25	50	0.02
320	312	299520	52651	17.58	5700	1.90	949	0.32	103	0.03
340	294	299880	54768	18.26	6130	2.04	1082	0.36	142	0.05
360	277	299160	56876	19.01	6621	2.21	1221	0.41	155	0.05
380	263	299820	59231	19.76	7445	2.48	1400	0.47	188	0.06
400	250	300000	60891	20.30	7906	2.64	1507	0.50	236	0.08
1000	100	300000	98902	32.97	2716	9.24	10255	3.42	240	0.08
									3798	1.27

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Table 3.

Sequence Length	Total number wildtype sequences	Number of "T" forward failures	% "T" forward failures	Number of "T" reverse failures	% "T" reverse failures	Number of "C" forward failures	% "C" forward failures	Number of "C" reverse failures	% "C" reverse failures
40	2500	300000	120699	40	39139	13	11468	4	3993
60	1666	299880	132516	44	48716	16	16455	5	6426
80	1250	300000	142523	48	57790	19	22043	7	9600
100	1000	300000	150441	50	65780	22	27368	9	12556
120	833	299880	156926	52	73381	24	32832	11	16347
140	714	299880	163973	55	81099	27	38470	13	20145
160	625	300000	169582	57	87388	29	43686	15	23767
180	555	299700	173319	58	92501	31	48016	16	27141
200	500	300000	178247	59	98957	33	53122	18	30973
220	454	299640	181728	61	103638	35	57622	19	34684
240	416	299520	184465	62	107959	36	62015	21	38330
260	384	299520	188025	63	112626	38	65898	22	41402
280	357	299880	191722	64	117075	39	70162	23	45504
300	333	299700	194210	65	120601	40	73900	25	48553
320	312	299520	196727	66	124257	41	77929	26	51963
340	294	299880	199290	66	127979	43	81308	27	54874
360	277	299160	200599	67	131116	44	84764	28	58213
380	263	299820	203752	68	134422	45	87956	29	60909
400	250	300000	205749	69	137686	46	91319	30	64056
420	238	299880	207767	69	140874	47	94747	32	67463
440	227	299640	209037	70	143182	48	96836	32	69713
460	217	299460	210530	70	145413	49	99414	33	71853
480	208	299520	212472	71	147958	49	102503	34	74722
500	200	300000	214285	71	149928	50	104741	35	77298
520	192	299520	215159	72	152227	51	107376	36	79507
540	185	299700	215902	72	153784	51	109511	37	81602

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TABLE 4

EXPECTED TUB FRAGMENTS		FRAGMENTS NOT SEEN
GGC	1045.6	
CCAC	1198.8	
CCACA [SEQ ID NO:22]	1512*	
CCAG	1318.8	
GGAC	1358.8	
CCAGCCG [SEQ ID NO:23]	2226.4	
GCGCAAG [SEQ ID NO:24]	2210.4	
GCGCAAGA [SEQ ID NO:25]	2523.6*	
CCGCGAAC [SEQ ID NO:26]	2539.6	
GGAGCACGCAGG [SEQ ID NO:7]	3880.4	
CGGCAAGCACCGACAGG [SEQ ID NO:8]	5374.4	
GGTGACCTGAACCACCTCGTGC [SEQ ID NO:9]	5888.8	PRIMER
CAGGCGCTTGAGACTGGACGCGT [SEQ ID NO:10]	6258	PRIMER
EXPECTED TUB-M FRAGMENTS		
CCAC	1198.8	END
CGAG	1358.8	
GGAC	1358.8	
CGAGGC [SEQ ID NO:27]	1977.2	
CCAGCCG [SEQ ID NO:28]	2226.4	
GCGCAAG [SEQ ID NO:29]	2210.4	
CGACAGCC [SEQ ID NO:30]	2539.6	
CCGCGAAC [SEQ ID NO:31]	2539.6	
CGAACGGC [SEQ ID NO:32]	2579.6	
GGAGCACGCAGG [SEQ ID NO:11]	3880.4	
GGTGACCTGAACCACCTCGTGC [SEQ ID NO:12]	5888.8	PRIMER
CAGGCGCTTGAGACTGGACGCGT [SEQ ID NO:13]	6258	PRIMER

* Fragments obtained due to the terminal transferase activity of Taq polymerase which results in the addition of a dATP at the 3' end of the PCR product.

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EXAMPLE 10
MODIFICATION DETECTION PROTOCOL

The method of Example 8 is employed except DNA polymerase enzymes are employed with the ability to incorporate both dNTPs and rNTPs. Specific cleavage reactions are performed on PCR products in which one of the nucleotides is substituted for rNTP. This permits the base specific cleavage reactions to be conducted in alkali at high temperature.

EXAMPLE 11
IDENTIFICATION OF MUTATION POSITION

The method of Example 8 employs Uracil-N-glycosylase which cleaves DNA at uracil. It is, therefore, a T reaction as uracil is replacing thymidine in the PCR product. In this Example, cleavage occurs at each of other bases so as to create sets of overlapping data to give information about the position of the mutation.

EXAMPLE 12
DETERMINATION OF NUCLEOTIDE SEQUENCE

The method of the present invention is used to determine a nucleotide sequence of a nucleic acid fragment. The method employed is substantially as described in Example 8.

EXAMPLE 13
DETECTION OF PREVIOUSLY UNKNOWN MUTATIONS

The method of the present invention is further demonstrated on a sequence polymorphism in the IL-12 gene. This previously unreported sequence change results in a TaqI RFLP and, therefore, can be followed by enzymatic digestion of PCR products.

Methods

Template DNA was genomic DNA from human volunteers of each possible genotype of the IL-12 polymorphism (ie. +/+, +/-, and -/-, where + is the presence of the Taq restriction site). PCRs were carried out in 20 μ l reactions in 192 well plates in a Corbett Thermocycler with the following reaction mixture: 50mM KCl, 10mM Tris-HCl pH 8.3, 25mM MgCl₂, 2.5mM dATP, dCTP and dGTP (Promega), 5mM dUTP (Boehringer Mannheim GmbH), 0.5U AmpliTaq Gold (Perkin Elmer), 0.4 μ M primers (Bresatec). After an initial 15min incubation at 95°C, the reactions were cycled 95°C 15 secs, 58°C 35sec, 72°C 35sec, for 40 cycles. 7 reactions were pooled for the homozygotes and 9 for the heterozygote. 1 unit of AmpErase Uracil-N-glycosylase (Perkin Elmer) was added to each pool and the reaction incubated at 50°C for 1 hour, followed by 30 minutes at 105°C. The extend of completion of the cleavage reaction was monitored by the absence of a band on an agarose gel. The cleavage reaction was monitored by the absence of a band on an agarose gel. The cleavage products were purified using reverse phase HPLC on a 100x2.1mm C8 aquapore RP300 column (Applied Biosystems). The flow rate was 0.5ml/min and absorbance was monitored at 254nm. The sample washed with 0.1M triethylaminoacetate (TEAA) and eluted in 0.1M TEAA/60% w/v acetonitrile and the fraction with absorbance at 254nm was collected and evaporated to dryness using a Savant Speedivac. The residue was resuspended in 100 μ l distilled deionised water and evaporated to dryness and then resuspended in 1 μ l water. 0.5 μ l of this was mixed with 0.5 μ l 3-hydroxypicolinic acid (saturated solution in 50% w/v acetonitrile and 0.5 μ l NH₄⁺ ion-exchange beads (BioRad, 50W-X4, mesh size 100-200 μ m) on a sample slide. The mass spectrometer used to characterise the reaction products was a Voyager BioSpectrometry Workstation from PerSeptive Biosystems. 128 laser pulses at power 1800 were averaged. Post Source Decay spectra were collected using a Kratos Kompact MALDI4 TOF mass spectrometer with 377nm laser and a curved field reflector in positive ion mode. Matrix and sample preparation as above. After scanning in linear mode for the sweet spot, the ion gate was set 34.8 Da above and 36.2 Da below the parent ion at 1727.2 Da. 200 profiles at 5 shots per profile were averaged. Spectra were corrected for the curved field.

Genotypes were confirmed by demonstrating the presence or absence of the TaqI restriction site

by digesting PCR products with TaqI restriction enzyme (Gibco-BRL) and analysing the products by agarose electrophoresis. DNA bands were stained with ethidium bromide.

A computer simulation of the method has been written and 100kb of random coding sequence from Genbank has been fed into it. The program takes discrete-length bites of sequence from a file of concatenated cDNA sequence from Genbank. Each base is mutated to each hypothetical variant of the original sequence by removing the cleaved base leaving the residual short strings. The mass spectrometry was modelled, fragments of different nucleotide composition being distinguishable and those of identical composition being indistinguishable. As quantitation is difficult on the MALDI, changes in peak height was not used as an indication of a change in underlying sequence. The program then compares "spectra" and tallies the number of mutations that were missed. The program can model the detection of a mutation in the presence of a wild-type sequence (heterozygote) or can model the differences between two homozygotes. In the first case a mutation can only be detected by the presence of a new peak and in the latter case, as well as the presence of a new peak, the disappearance of a peak can also signal a change. All four base specific cleavage reactions were used and reactions were performed on separated strands giving a total of 8 reactions per PCR product. Also the model has been refined to take account of the ability of post source decay (PSD) to identify changes in peaks containing a complex mix of oligonucleotides. In this case fragments of different sequence are distinguishable.

Results

A PCR assay was designed to incorporate the mutated region and then subjected to uracil -N glycosylase treatment. The products were purified and analysed by MALDI-TOF mass spectrometry. The sequence of the PCR primers and product along with the mutation are shown in Figure 4. The C to T change gives rise to a Taq RFLP and this can be seen in homozygote and heterozygote state in Figure 5. The spectra generated by the MALDI-TOF can also be seen in Figure 5. The expected and observed masses of the cleavage products from the two alleles are given in Table 5. The position of the mutation and deduction of the changed base is evident from study of this Table.

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A limitation to the sensitivity of this method results from the lack of quantitative data available from the MALDI. When the fragment derived from the mutated sequence coincides with other fragments of identical nucleotide composition in the wild-type sequence, its disappearance will go undetected. Similarly, the appearance of a new fragment in the mutated sequence will go unnoticed if it has identical nucleotide composition to one or more other cleavage products. If both these conditions exist for all cleavage reactions, then the mutation will be missed. This technique, therefore, is not as advantageous for longer fragment as for small fragments.

To address this problem, the inventors employed a second dimension detection protocol on the MALDI-TOF machine. Post source decay (PSD) uses the dissociation of the highly energised ions during their flight to the detector as this second dimension. They are directed into an electric field of opposite polarity and are reflected. The smaller ions are reflected earlier and reach the detector first. As the spectrum from the decay is dependent on the sequence of the oligonucleotide (and not the nucleotide composition), the aforementioned limitation is bypassed, generating a method of mutation detection that is now extremely sensitive.

The utility of MALDI-TOF analysis with PSD is demonstrated in Figure 6 where two oligonucleotides of identical nucleotide composition are separated by MALDI-TOF using PSD. The resulting spectra are quite distinguishable. Sequence determination of small oligonucleotides is feasible using molecular dissociation methods and, therefore, the subject method extrapolates into an accurate resequencing protocol.

A computer simulation of data from the linear separation of cleavage products has been written. Using Genbank data, the expected number of base substitution that would be identified when comparing two homozygotes over a 250bp PCR distance is 98.5%. the comparable figure is 95% when a homozygote is compared to a heterozygote. If each mass peak from a base specific cleavage is analysed using a secondary dissociation technique, eg. PSD on the MALDI-TOF machine, then sensitivity of mutation detection improves dramatically. This has also been simulated and for a 1000bp fragment subjected to base specific cleavage, and analysed with PSD, 99% of all substitutions will be detected for a homozygote to heterozygote comparison and 99.8% when two homozygotes are compared.

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Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(US only): FOOTE Simon, ELSO Colleen, SIMPSON Richard, REID Gavin

(ii) TITLE OF INVENTION: A NOVEL METHOD

(iii) NUMBER OF SEQUENCES: 32

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(D) STATE: VICTORIA
(E) COUNTRY: AUSTRALIA
(F) ZIP: 3000

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT INTERNATIONAL
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(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: PO 7102
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(ix) TELECOMMUNICATION INFORMATION:

- 33 -

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- (C) TELEX: AA 31787

- 34 -

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTC**A**TUTTT TUTTG**T**AGA GG

22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTC**G**TUTTT TUTTG**T**UAGA GG

22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGTGACCTGA ACCACCTCGT GCGTCCAGCC GTTCGTGGCT GTCCAGTCCG
CAAACCTCTGA CCTGCGCAAG

50

70

- 35 -

(2) INFORMATION FOR SEQ ID NO:4:

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(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGTGACCTGA ACCACCTCGT CGTCCAGCCG TTTCGAGGCTG TCGAGTCCGC
(G) AACTCTGAC CTGCGCAAG

50

69

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGACCTGA ACCACCTCGT

20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTGGCGCAGG TCAGAGTT

18

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAGCACCGCAG G

11

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGCAAGCAC CGACAGG

17

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTGACCTGA ACCACCTCGT GCG

23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- 37 -

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGGCGCTTG AGACTGGACG CGT

23

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGAGCACGCA GG

12

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTGACCTGA ACCACCTCGT GCG

23

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

- 38 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAGGCGCTTG AGACTGGACG CGT

23

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 124 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAACGGAA TAGACCCAAA AAGAUUUU CUAUCUGAUU UGCUUUAAAA
 CGUUUUUUUA GGAUCACAAU GAUAUCUUUG CUGUAUUUGU AUAGUUCGAU
 GCUAAAUGCU CAUUGAAACA AUCA

50

50

24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 124 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GUGUUGCCUU AUCUGGGUUU UUCUAUUAAA GAUAGACUAA ACGAAAUUU
 GCAAAAAAAAU CCUAGUGUUA CUAUAGAAC GACAUAAACA UAUCAGCUA
 CGATTTACGA GTAACTTGT TAGT

50

50

24

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 base pairs
- (B) TYPE: nucleic acid

- 39 -

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CATCCT

6

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CACCTT

6

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCTCAT

6

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- 40 -

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTGTAAGAGG

10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCTCGT

6

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGAGG

5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCACCA

5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCAGCCG

7

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCGCAAG

7

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GCGCAAGA

8

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(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CCGCGAAC

8

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CGAGGC

6

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CCAGCCG

7

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 base pairs

- 43 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GCGCAAG

7

(2) INFORMATION FOR SEQ ID NO: 30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

CGACAGCC

8

(2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CCGCGAAC

8

(2) INFORMATION FOR SEQ ID NO: 32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CGAACGGC

8

CLAIMS:

1. A method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.
2. A method according to claim 1 wherein the nucleic acid molecule to be tested is amplified by a polymerase chain reaction (PCR) prior to base specific cleavage.
3. A method according to claim 1 or 2 wherein the base specific cleavage results in oligonucleotide fragments of from about 2 bases to about 1000 bases.
4. A method according to claim 3 wherein the base specific cleavage results in oligonucleotide fragments of from about 3 bases to about 500 bases.
5. A method according to claim 4 wherein the base specific cleavage results in oligonucleotide fragments of from about 4 bases to about 100 bases.
6. A method according to any one of claims 1 to 5 wherein the base specific cleavage is uracil specific cleavage.
7. A method according to claim 6 wherein the uracil specific cleavage is mediated by uracil-N-glycosylase.
8. A method according to any one of claims 1 to 7 further comprising subjecting

fragmentation products to further separation (PSD) to generate a spectrum from decay dependent on the nucleotide sequence of the oligonucleotide.

9. A method according to claim 8 wherein the further separation of fragmentation products is by post source decay (PSD).

10. A computer programme capable of controlling a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

11. A method according to claim 9 wherein the nucleic acid to be tested is amplified by PCR prior to base specific cleavage.

12. A method according to claim 9 or 10 wherein the base specific cleavage results in oligonucleotide fragments of from about 2 bases to about 1000 bases.

13. A method according to claim 9 wherein the base specific cleavage results in oligonucleotide fragments of from about 3 bases to about 500 bases.

14. A method according to claim 10 wherein the base specific cleavage results in oligonucleotide fragments of from about 4 bases to about 100 bases.

15. A method according to any one of claims 9 to 13 wherein the base specific cleavage is uracil specific cleavage.

16. A method according to claim 14 wherein the uracil specific cleavage is mediated by uracil-N-glycosylase.
17. A method according to any one of claims 10 to 16 further comprising the further separation of fragmentation products to generate a spectrum from decay dependent on the nucleotide sequence of the oligonucleotide.
18. A method according to claim 17 wherein the further separation of fragmentation products is by post source decay (PSD).
19. An apparatus capable of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said apparatus comprising means of subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.
20. An apparatus according to claim 19 further comprising further fragmentation separation means to generate a spectrum from decay dependent on the nucleotide sequence of the oligonucleotide.
21. An apparatus according to claim 20 wherein the further fragmentation separation means is post source decay (PSD).
22. Use of MALDI-TOF in the detection of a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule.
23. Use according to claim 22 further comprising use of PSD to generate a spectrum for

decay dependent on the sequence of an oligonucleotide.

24. A method for identifying and/or locating a mutation in one or more bases in a target nucleic acid molecule, subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.
25. A method according to claim 24 wherein the nucleic acid molecule to be tested is amplified by a polymerase chain reaction (PCR) prior to base specific cleavage.
26. A method according to claim 24 or 25 wherein the base specific cleavage results in oligonucleotide fragments of from about 2 bases to about 1000 bases.
27. A method according to claim 26 wherein the base specific cleavage results in oligonucleotide fragments of from about 3 bases to about 500 bases.
28. A method according to claim 27 wherein the base specific cleavage results in oligonucleotide fragments of from about 4 bases to about 100 bases.
29. A method according to any one of claims 24 to 28 wherein the base specific cleavage is uracil specific cleavage.
30. A method according to claim 29 wherein the uracil specific cleavage is mediated by uracil-N-glycosylase.
31. A method according to any one of claims 24 to 30 further comprising subjecting fragmentation products to further separation (PSD) to generate a spectrum from decay

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dependent on the nucleotide sequence of the oligonucleotide.

32. A method according to claim 31 wherein the further separation of fragmentation products is by post source decay (PSD).

ABSTRACT

A method of detecting a mutation or a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-ATOF MS and/or other equivalent procedure to produce a fingerprint of then oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

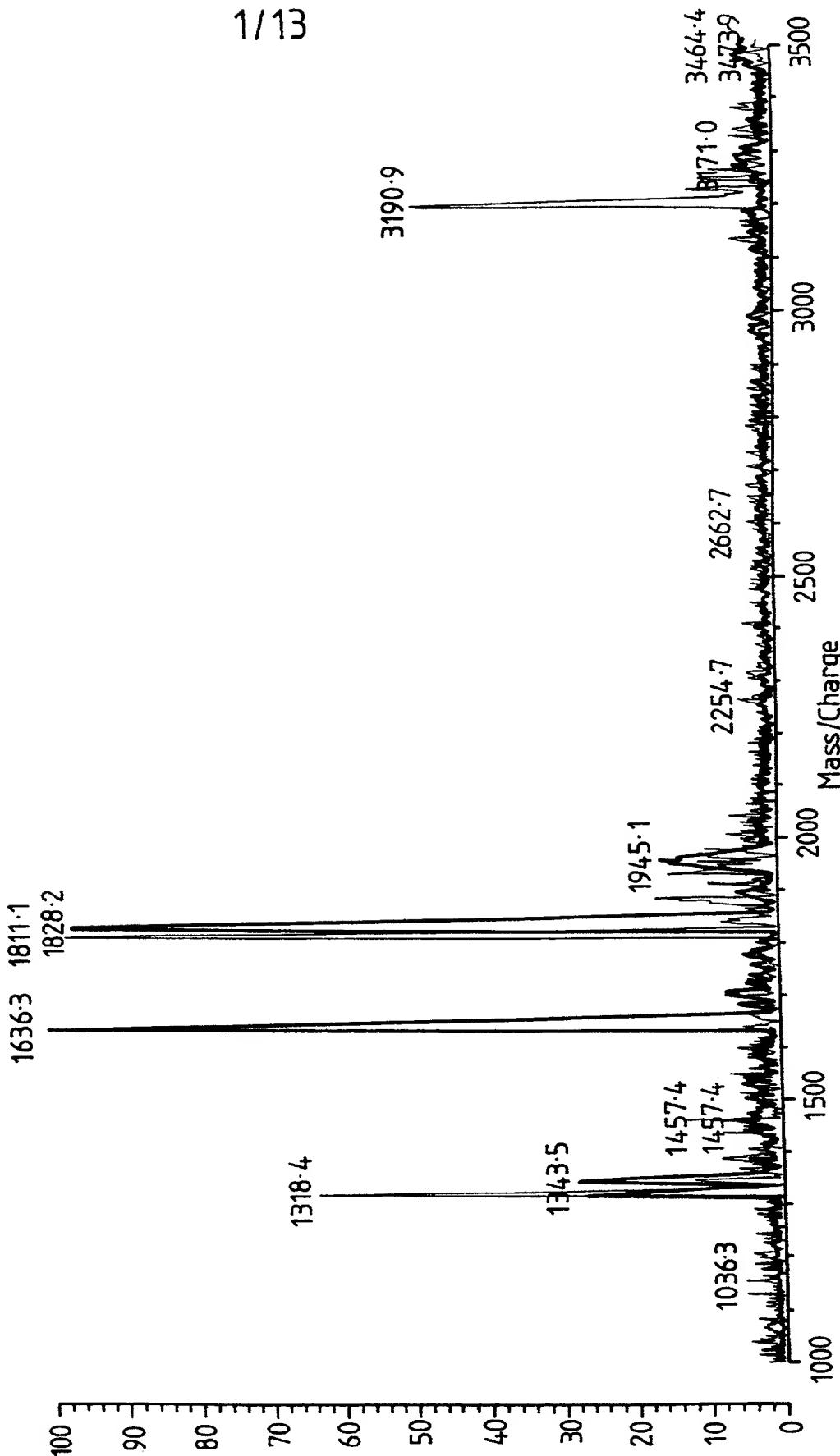
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FIG 1



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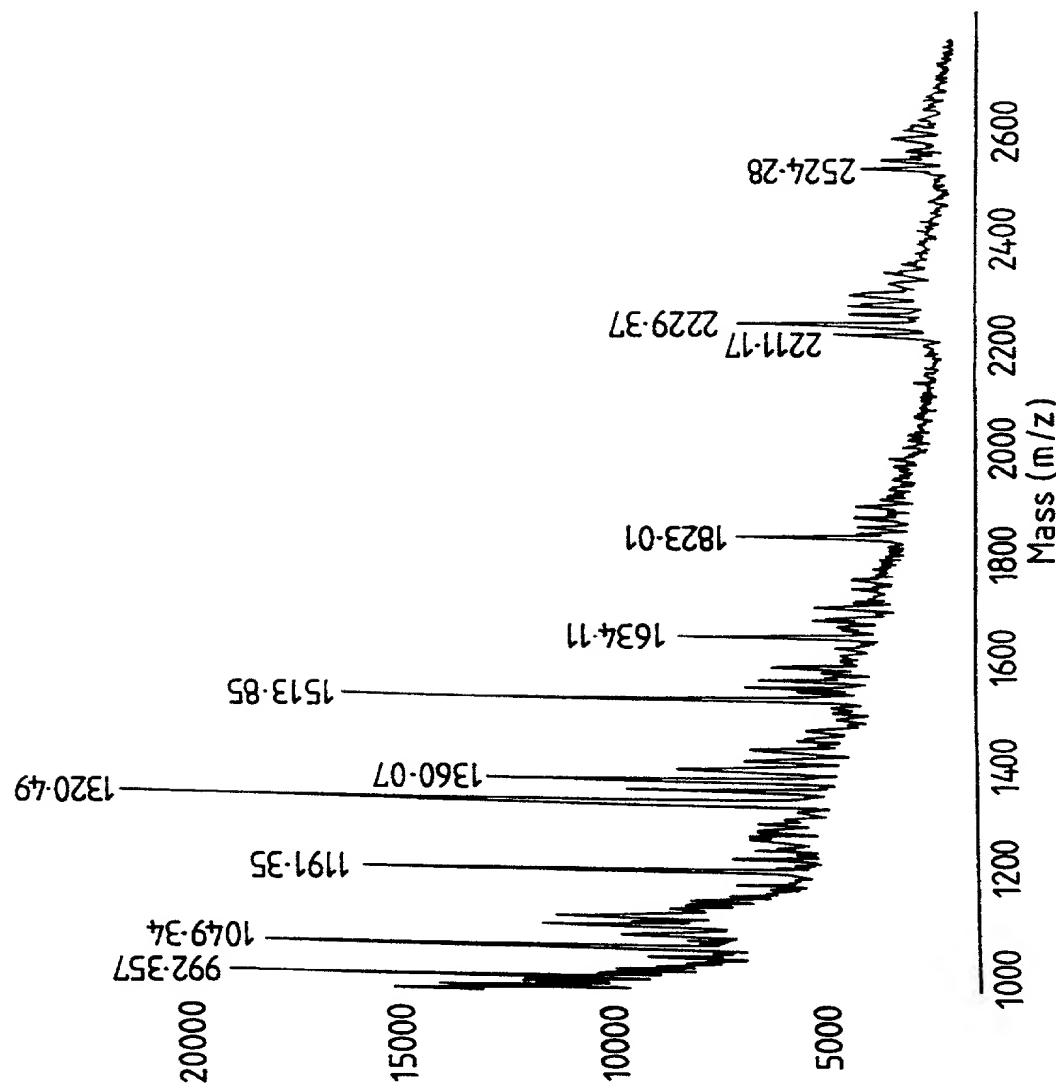


FIG 2

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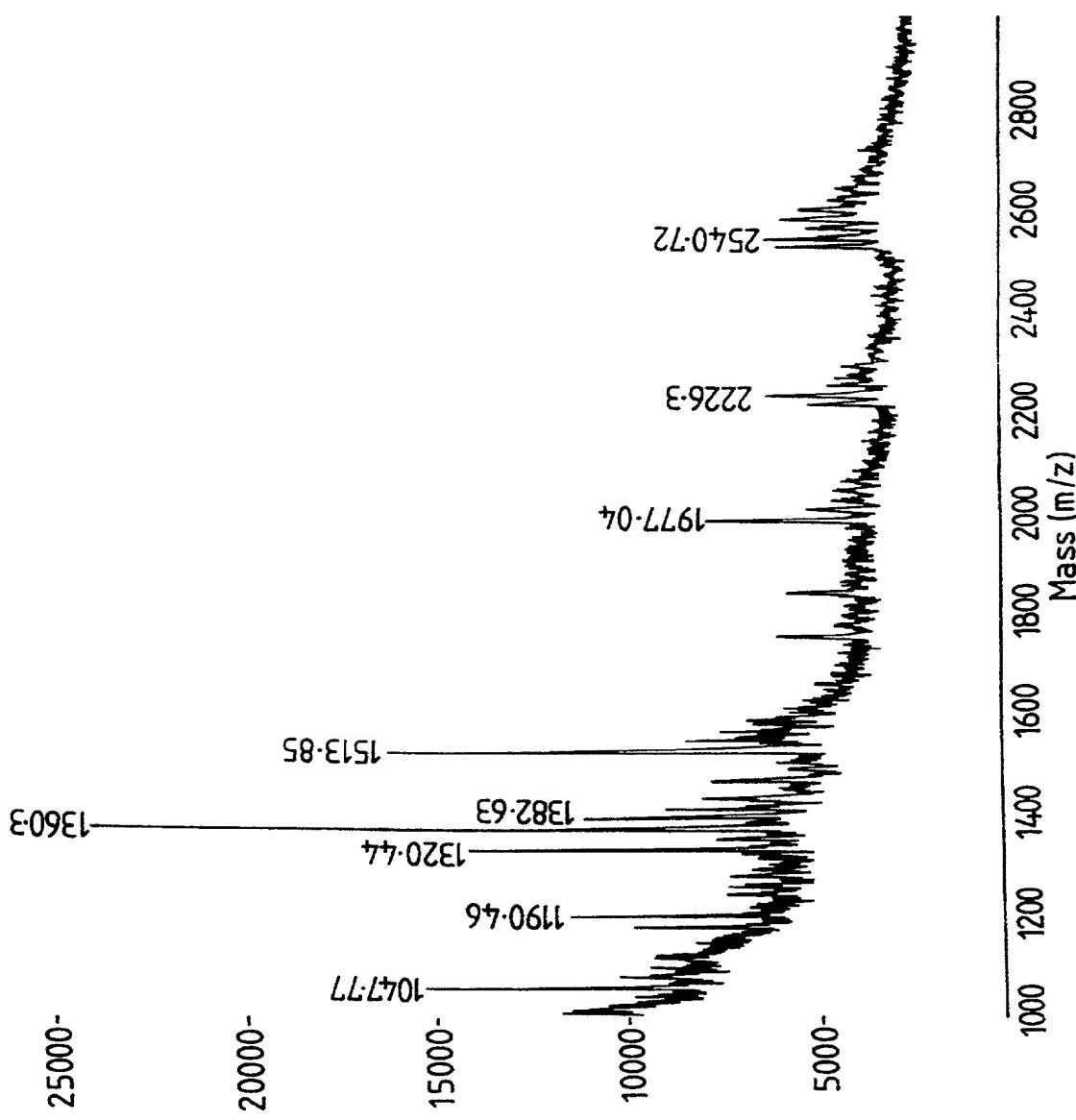


FIG 3

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CAC AAC GGA ATA GAC CCA AAA AGA UAA UUU CUA UCU
GUG UUG CCU UAU CUG GGU UUU UCU AUU AAA GAU AGA

GAU UUG CUU UAA AAC GUU UUU UUA GGA UCA CAA UGA
CUA AAC GAA AUU UUG CAA AAA AAU CCU AGU GUU ACU

UAU CUU UGC UGU AUU UGU AUA GUU CGA UGC UAA AUG
AUA GAA ACG ACA UAA ACA UAU CAA GCU ACG ATT TAC *

CUC AUU GAA ACA AUC A
GAG TAA CTT TGT TAG T

FIG 4

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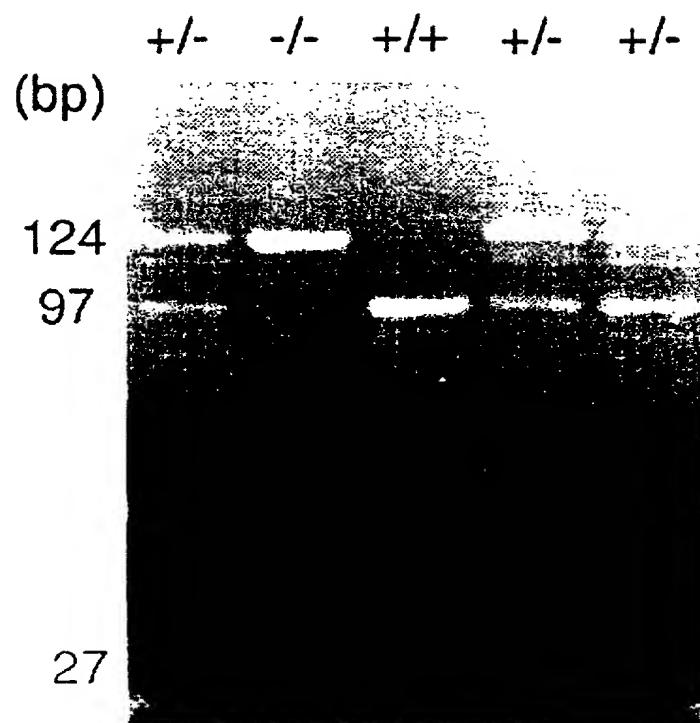
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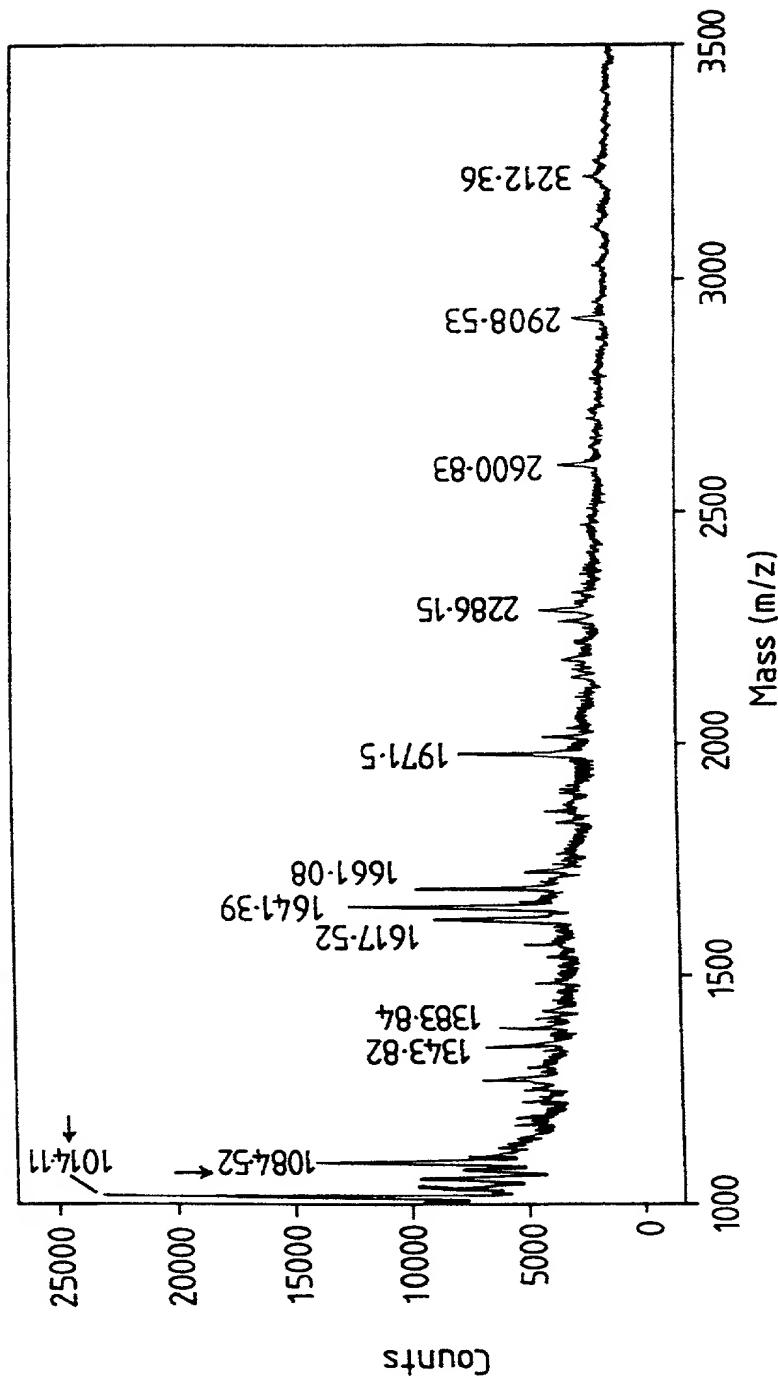
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Figure 5A

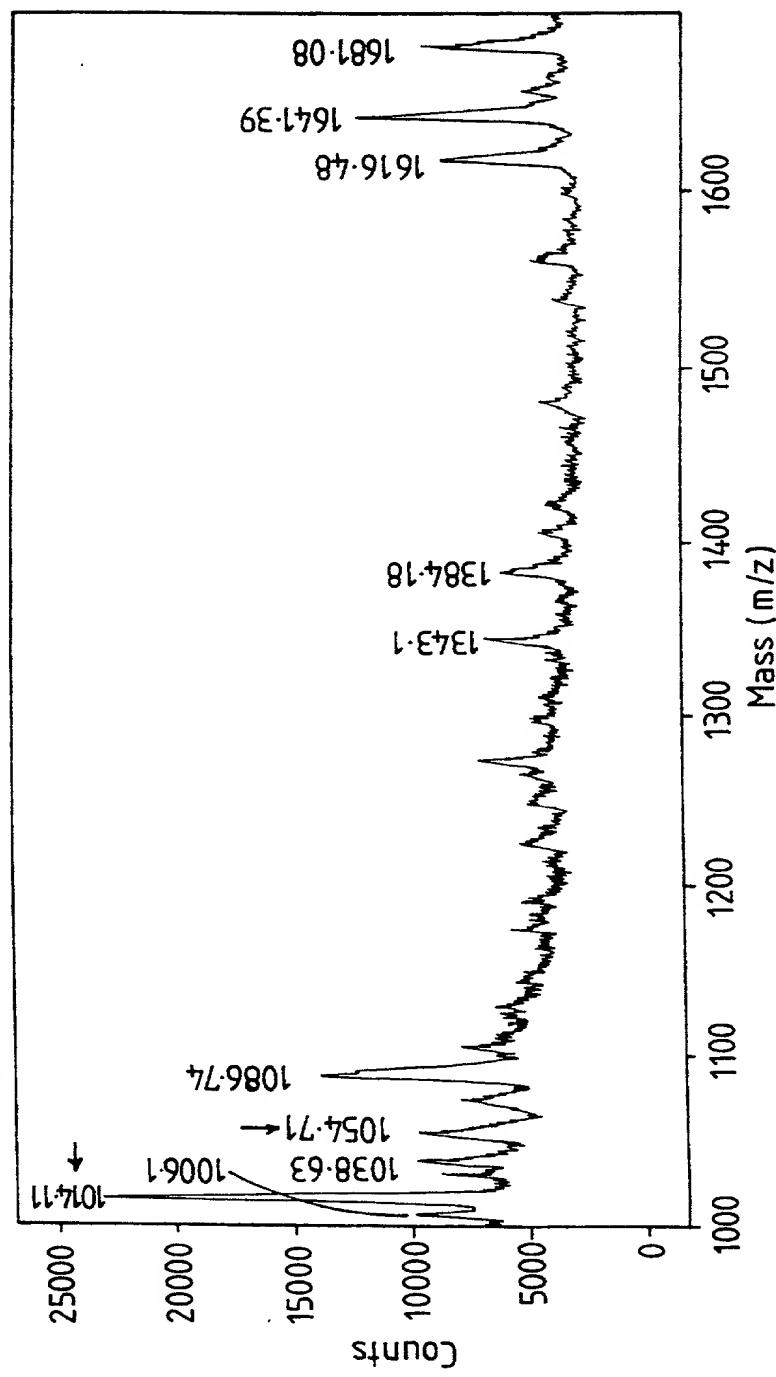
A



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FIG 5B (i) a

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FIG 5B (i)b

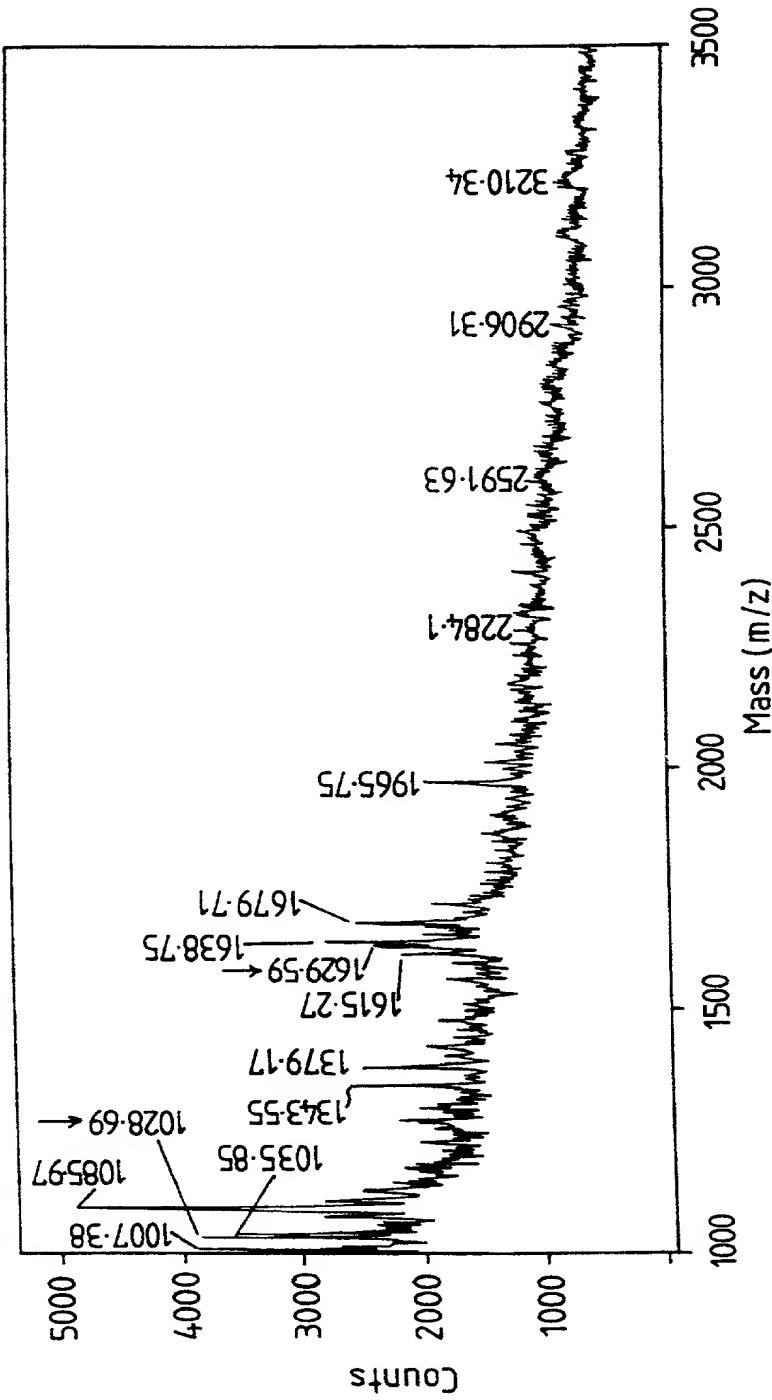
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FIG 5B (ii) a



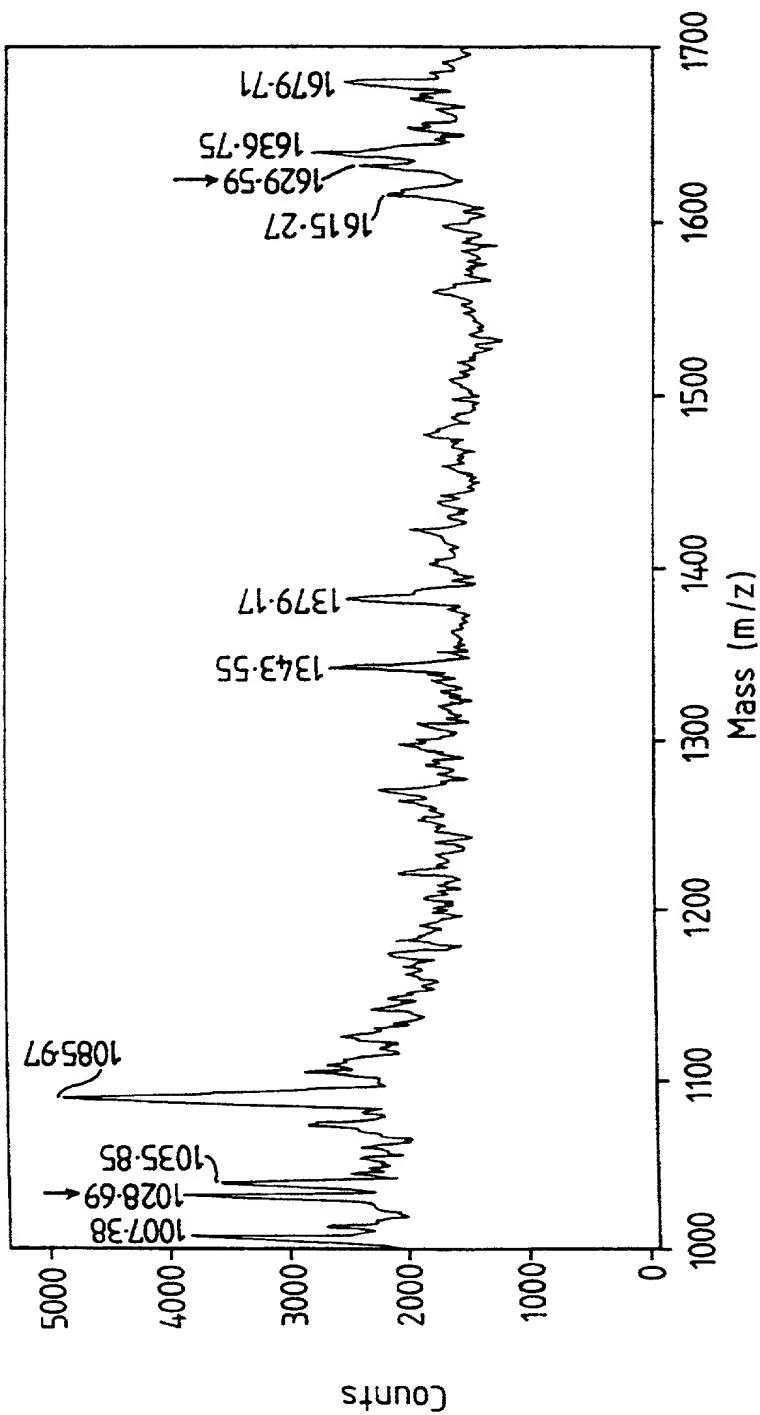
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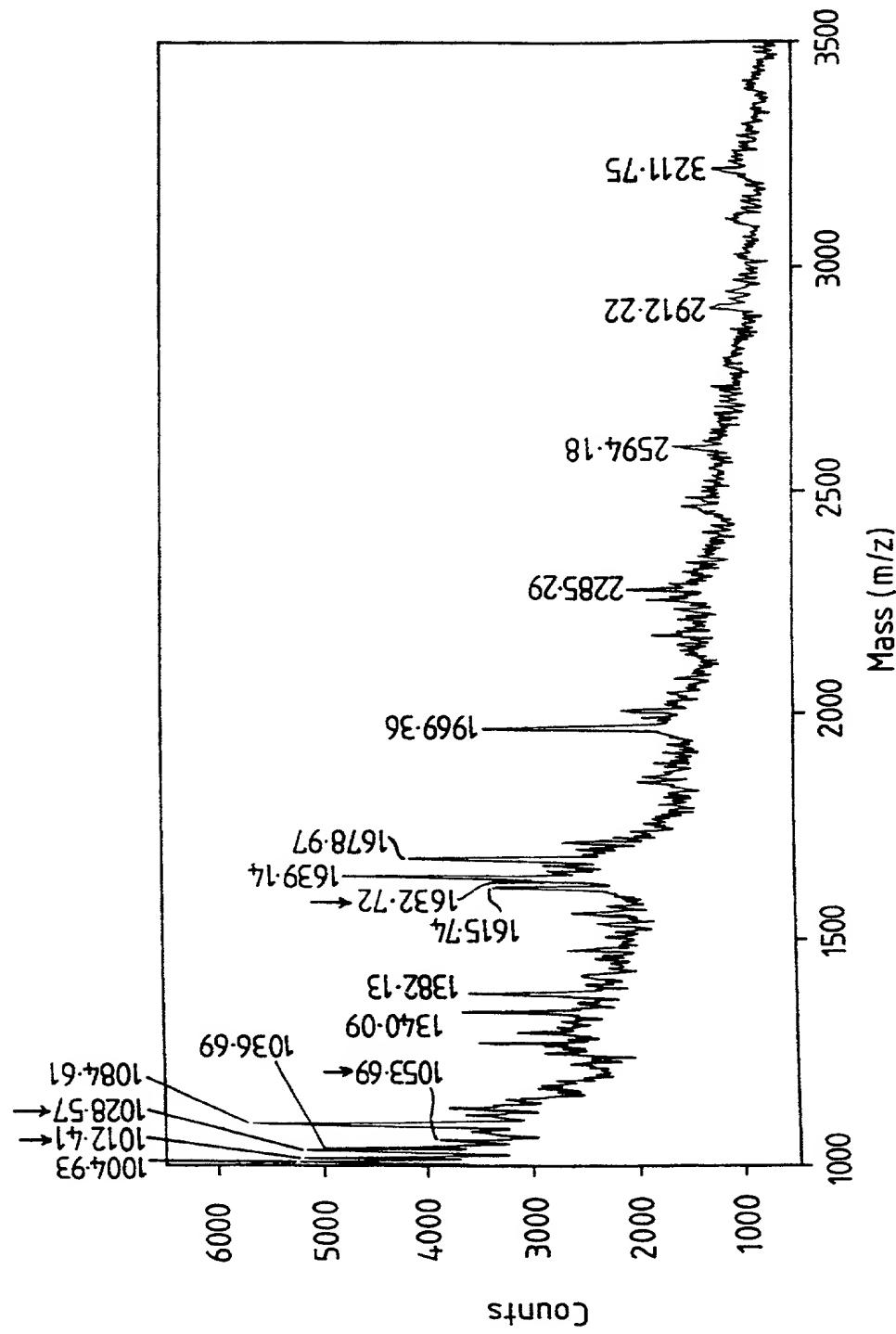
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FIG 5B (ii)b



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FIG 5B (iii)a

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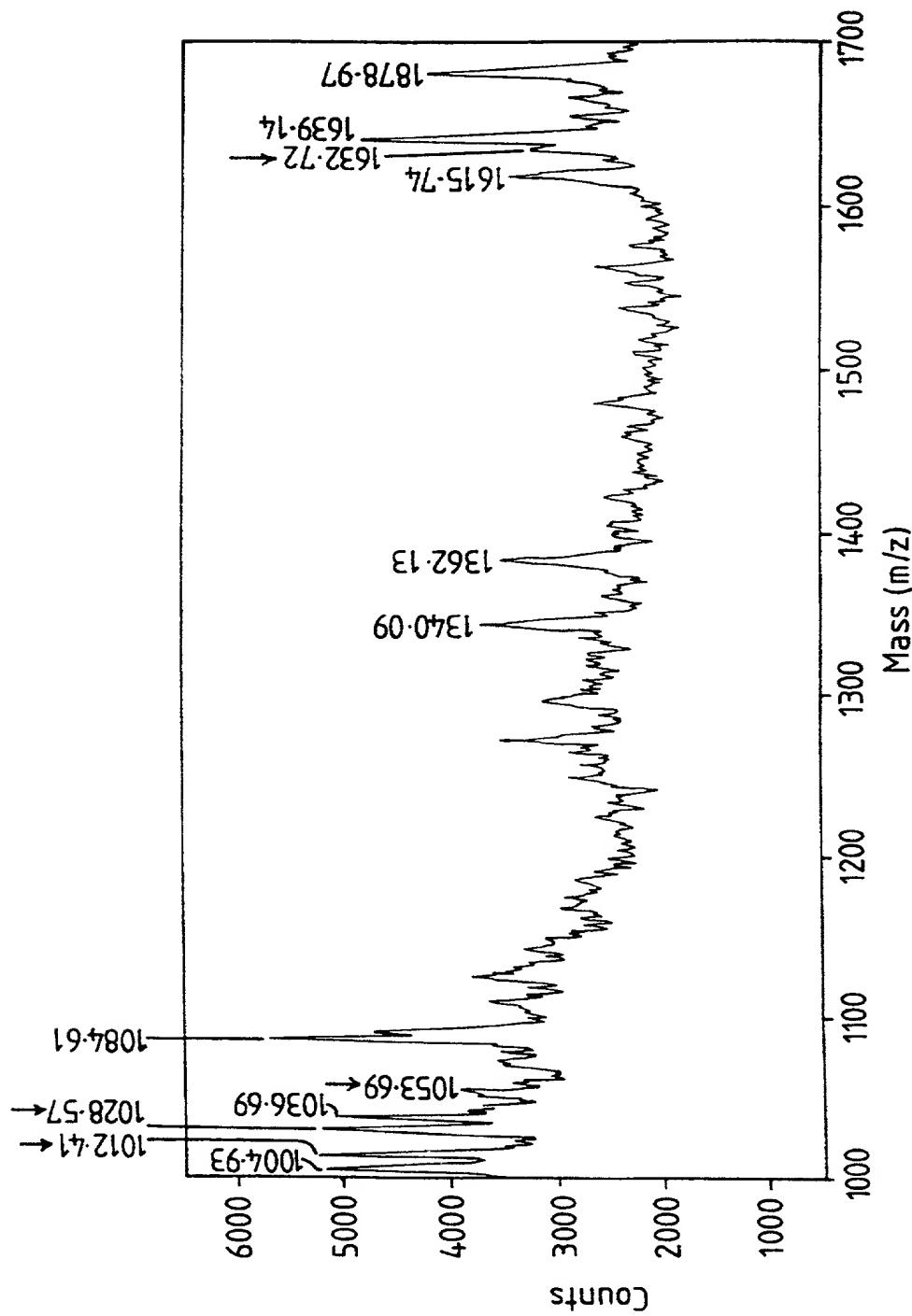


FIG 5B(iii)b

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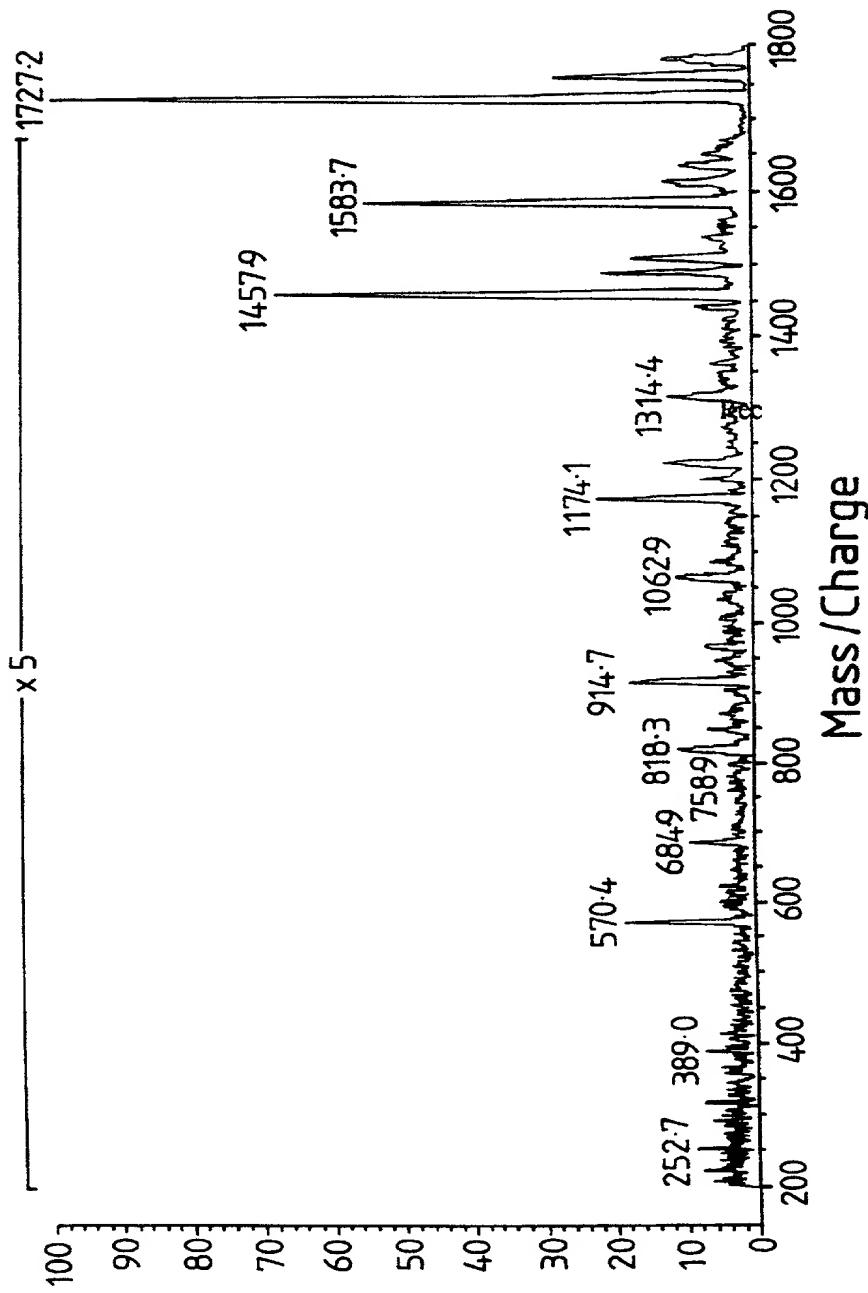


FIG 6A

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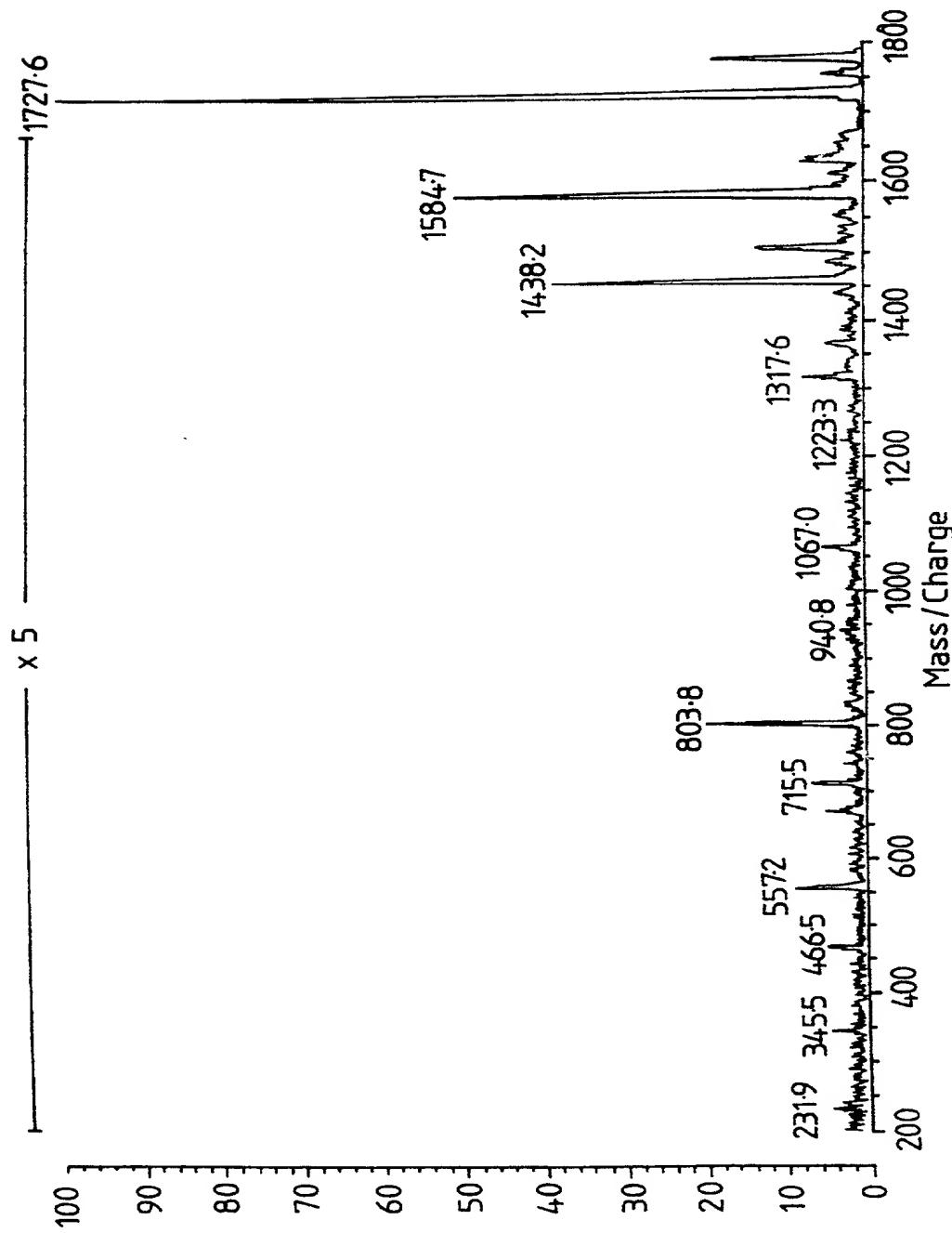


FIG 6B

COMBINED DECLARATION FOR PATENT APPLICATION AND TRADE ATTORNEY



As a U.S. citizen or resident:

My residence, post office address and citizenship are as set forth below my name.

I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if two names are listed below) of the subject matter which is claimed and for which a patent is sought in the invention entitled:

"Nucleic acid diagnostics based on mass spectrometry or mass separation and base specific cleavage"

the specification of which I check only one item below:

is attached hereto
 is filed as United States application

Serial No.

(or)

and was amended

on _____

(if applicable)

was filed as PCT International application

Number PCT/AU98/00396

on 28 May 1998

and was amended under PCT Article 19

on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119

Country	Application No.	Date Filed	Status
Australia	PO 7102	28 May 1997	X ..
Australia	PO 7109	30 May 1997	X ..
Australia	PP 1665	5 February 1998	X ..
Australia	PP 3592	19 May 1998	X ..
			□ ..

Continued Declaration - Patent Application No. 09/522,110

I, Scott Scully, do hereby declare that I am the inventor(s) of the subject matter described in the specification which follows, and that it has not been previously disclosed to the public of the United States or elsewhere by me or another person as defined in Title 35, Code of Federal Regulations, 37, which occurred before the date of the filing of this application or PCT International Application Date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER
35 U.S.C. 120

APPLICATION NUMBER		FILING DATE	
PCT/AU98/00396	28 May 1998		

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *10*
Stephen D. Murphy, Reg. No. 22,002; Leopold Presser, Reg. No. 19,827; William C. Koch, Reg. No. 21,972; Kenneth L. King, Reg. No. 21,223; Frank S. DiGiglio, Reg. No. 31,348; Paul J. Esatto, Jr., Reg. No. 20,741; John S. Sonany, Reg. No. 28,757; Mark J. Cohen, Reg. No. 32,211; Richard L. Catania, Reg. No. 32,608 and Donald T. Black, Reg. No. 27,999.

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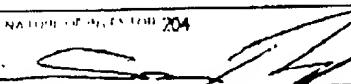
I declare under penalty of perjury that the statements made before me are true to the best of my knowledge and that all statements made in this declaration and before the USPTO are true, and further that if these statements were made with the knowing and intended intent to deceive, they would be punishable by law as imprisonment or both under Title 18, United States Code, and that such wilful false statements may jeopardize the validity of the application or patent it covers.

<i>10</i>	<i>Colleen ELSO</i>	<i>R. Presser</i>
<i>17/02/00</i>	<i>17/2/00</i>	<i>18/2/00</i>

[] Signature for fourth and subsequent joint inventors.
Number of pages added _____.

204	REID	Gavin	
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FULL NAME OF INVENTOR	NAME	JOHN GIVEN NAME	COUNTRY OF CITIZENSHIP
RESIDENCE & CITIZENSHIP	CITY	STATE & ZIP CODE/COUNTRY	STATE & ZIP CODE/COUNTRY
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & GIVEN NAME
FULL NAME OF INVENTOR	NAME	JOHN GIVEN NAME	COUNTRY OF CITIZENSHIP
RESIDENCE & CITIZENSHIP	CITY	STATE & ZIP CODE/COUNTRY	STATE & ZIP CODE/COUNTRY
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RESIDENCE & CITIZENSHIP	CITY	STATE & ZIP CODE/COUNTRY	STATE & ZIP CODE/COUNTRY
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & GIVEN NAME

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 204	SIGNATURE OF INVENTOR 205	SIGNATURE OF INVENTOR 206
		
DATE 17 February 2000	DATE	DATE
SIGNATURE OF INVENTOR 207	SIGNATURE OF INVENTOR 208	SIGNATURE OF INVENTOR 209
DATE	DATE	DATE